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Gene therapy for brain tumors: Regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo*

(herpes simplex virus thymidine kinase/ganciclovir)

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ABSTRACT **The therapeutic efficacy of adenovirus-mediated herpes simplex virus thymidine kinase (HSV-tk) gene transduction of rat C_6 glioma cells followed by ganciclovir (GCV) administration was studied in tumors generated in the brains of nude mice. C_6 glioma cells were efficiently transduced *in vitro* by a replicative-defective recombinant adenovirus carrying the HSV-tk gene (ADV/RSV-tk) that rendered them sensitive to GCV in a dose-dependent manner. Tumors were generated by stereotaxic intracerebral injection of 1×10^4 C_6 cells in nude mice. After 8 days of tumor growth, 3×10^8 ADV/RSV-tk viral particles were injected into the tumors and the mice subsequently were treated with GCV for 6 days. Tumor size in untreated and treated animals was compared 20 days after tumor implantation. The mean cross-sectional area of the tumors in the treated animals was 23-fold smaller than in control animals and the tumor volume was reduced by >500-fold. These results demonstrate that the recombinant adenoviral vector can function as an efficient gene delivery vehicle for the treatment of gliomas by *in vivo* gene therapy.**

Viral-mediated transfer of the herpes simplex virus thymidine kinase (HSV-tk) gene has been used to confer cytotoxic sensitivity to nucleoside analogues, such as ganciclovir (GCV), in a variety of tumor cells *in vitro* and *in vivo* (1–12). HSV-tk converts the nontoxic nucleoside analogue GCV into a phosphorylated compound that acts as a chain terminator in DNA synthesis, selectively killing dividing cells. This approach is especially suitable for treatment of solid tumors that are rapidly growing and invading normal tissue consisting largely of nonproliferating cells. Treatment with a recombinant retroviral vector containing HSV-tk caused regression of tumors generated by implanting 9L glioma cells in rat brains (9–11). This vector system was limited by low viral titer and low target cell transduction frequency. To attain effective viral titers, retrovirus-producing mouse fibroblasts were implanted into the target tumor tissues rather than the retrovirus itself. We have investigated the capacity of a recombinant adenoviral vector to produce a high level of transduction and gene expression and to render C_6 glioma cells susceptible to the toxic effects of phosphorylated GCV.

MATERIALS AND METHODS

Viral Constructs. The 2.8-kbp *Bgl* II/*Bam*HI fragment containing the HSV-tk gene and poly(A) tail was inserted into the *Bam*HI site of the plasmid pADL.1/RSV, which was obtained by insertion of the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter into the *Xba* I and *Cla* I sites of pXCJL.1 (kindly supplied by Frank Graham, McMaster University). In the resulting plasmid pADL.1/RSV-tk the HSV-tk gene is under the transcriptional control of RSV-LTR. To generate a recombinant adenovirus, pADL.1/RSV-tk and pJM17, a plasmid containing the complete adenovirus genome, were cotransfected into the 293 transformed human kidney cell line by calcium phosphate precipitation. Recombinant adenovirus was isolated from a single plaque, expanded in the 293 cell line, and purified by double cesium gradient ultracentrifugation as described (13). Virus titer was determined by optical absorbance at 260 nm.

***In Vivo* Experiments.** Athymic nude mice, 6–8 weeks old, were anesthetized with tribromoethanol (Avertin) (30 ml/kg) and placed in a stereotaxic frame (Stoelting). A burr hole was drilled in the skull 1 mm anterior and 2 mm lateral to the bregma with a 0.9 mm burr to expose the dura. Using a microliter syringe (Hamilton) fitted with a 26-gauge needle and connected to the manipulating arm of the stereotaxic frame, 1×10^4 C_6 glioma cells in 1 μl of Hanks' buffered saline were injected over 2.5 min into the caudate nucleus at a depth of 3.5 mm from the dura. The needle was left in place for 3 min and then withdrawn slowly over another minute.

The same coordinates were used for injection of virus except that the needle was placed 1 mm deeper than the tumor cells and the virus was injected at six points, 0.5 mm apart, along the needle track as the needle was withdrawn. The needle was coated with carbon particles (<30 μm) to mark the needle track and to verify colocalization of the virus injection with the tumor. A replication-defective recombinant adenovirus carrying the HSV-tk gene under transcriptional control of the RSV-LTR promoter (ADV/RSV-tk) or a replication-defective recombinant adenoviral vector containing the bacterial β-galactosidase (β-Gal) gene under transcriptional control of the RSV-LTR promoter (ADV/RSV-β-Gal) was used. ADV/RSV-tk or ADV/RSV-β-Gal (3×10^8 viral particles in 3 μl in 10% glycerol/1 mM $MgCl_2$/10 mM Tris·HCl, pH 7.0) was injected in a vol of 0.5 μl at each of the six positions over 10 min. The needle was left in the tissue for an additional 3 min and then was slowly withdrawn. The scalp wound was closed with Autoclips (Clay Adams). Twelve hours after the virus injections, the animals were divided into two groups. One group was treated intraperitoneally with GCV at a dose of 125 mg per kg of body weight twice daily for 3 days and then at 100 mg/kg for another 3 days. The other group was treated with PBS.

The mice were sacrificed 20 days after tumor injection and fixed by intracardial perfusion of 4% paraformaldehyde in

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Abbreviations: HSV-tk, herpes simplex virus thymidine kinase; GCV, ganciclovir; RSV-LTR, Rous sarcoma virus long terminal repeat; β-Gal, β-galactosidase; ADV/RSV-tk, replication-defective recombinant adenovirus carrying the HSV-tk gene under transcriptional control of the RSV-LTR promoter; ADV/RSV-β-Gal, replication-defective recombinant adenoviral vector containing the bacterial β-Gal gene under transcriptional control of the RSV-LTR; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; moi, multiplicity of infection.

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PBS. The brains were rem ved, cryoprotected in 21% sucrose in PBS, and frozen in OCT. Coronal sections (10 μm) were taken from the tumor implantation site and stained with hematoxylin and eosin. Image analysis was performed by using Bi scan Optimas s ftware (Edm nds, WA) and tumor sizes between groups were analyzed by ANOVA.

RESULTS

Quantitative Transduction of C_6 Glioma Cells *in Vitro* by Recombinant Adenovirus. Adenovirus transduction of C_6 cells *in vitro* was tested by using ADV/RSV-β-Gal (14). The C_6 cell line was originally derived from a rat glial tumor induced by *N*-nitrosomethylurea (15). As demonstrated by 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining of β-Gal activity, ADV/RSV-β-Gal transduced the C_6 glioma cells at 100% efficiency *in vitro* at a multiplicity of infection (moi) >125 (Fig. 1).

Cytopathicity of GCV in HSV-tk Transduced C_6 Glioma Cells. To determine whether introduction of the HSV-tk gene would render C_6 cells susceptible to killing by GCV, ADV/RSV-tk was constructed. This recombinant adenovirus was used to transduce C_6 glioma cells in culture. The viral tk activity in cellular extracts was determined by [^{3}H]acyclovir phosphorylation (16). The HSV-tk gene was expressed at high levels in transduced C_6 cells and saturation was achieved after transduction at a moi of 1000 (Fig. 2*A*). The reason that activity continued to increase at a moi >125, where 100% cell transduction was achieved, was probably the result of multiple cellular transduction events. To test whether C_6 glioma cells expressing the HSV-tk gene were susceptible to GCV toxicity, the transduced cells were treated with either PBS or 10 μg of GCV per ml of culture medium (Fig. 2*B*). A moi of 300 and above resulted in >90% cell death. There was no cytotoxic effect of GCV on cells transduced with the control virus ADV/RSV-β-Gal.

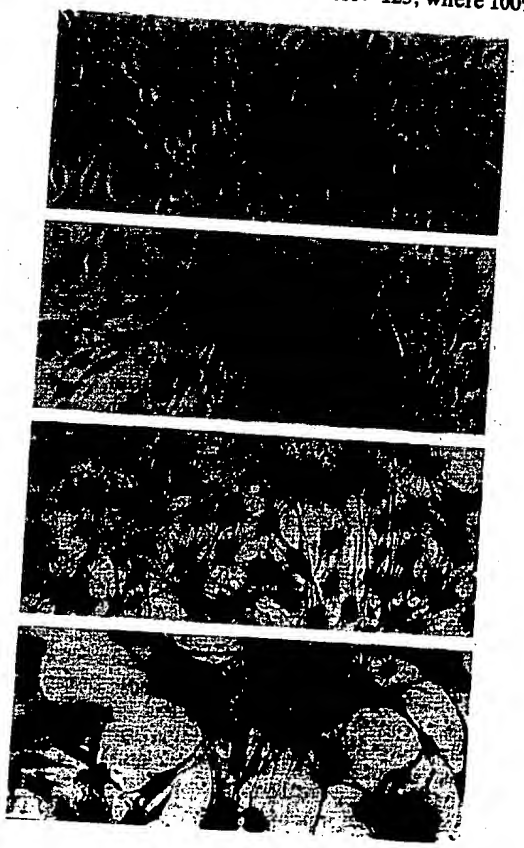

FIG. 1. Transduction efficiency of adenovirus in C_6 glioma cells *in vitro* was demonstrated by using ADV/RSV-β-Gal. The β-Gal enzyme in this construct contains a nuclear translocation signal and results in dense nuclear staining. C_6 cells (5×10^5) were plated on 1.5-cm-diameter wells, transduced with ADV/RSV-β-Gal at various viral doses, and stained with X-Gal 48 hr later. *A*, moi = 0; *B*, moi = 125; *C*, moi = 500; *D*, moi = 2000.

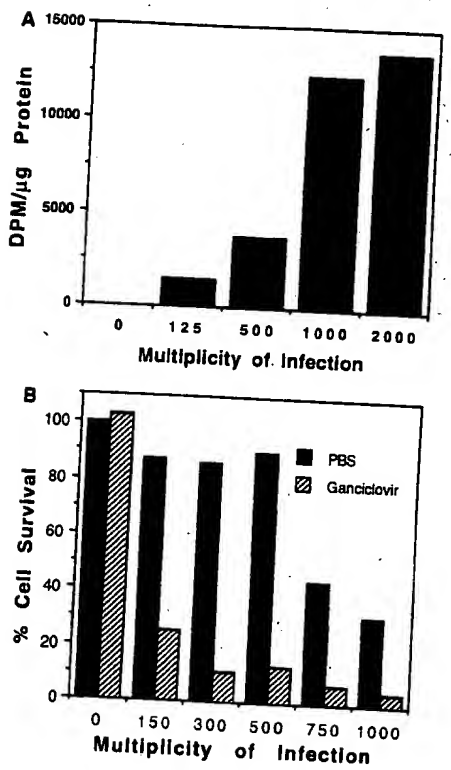

FIG. 2. Transduction of C_6 glioma cells with ADV/RSV-tk *in vitro*. (*A*) Expression of HSV-tk gene in C_6 glioma cells after transduction by ADV/RSV-tk. Cells (5×10^6) were plated on 1.5-cm-diameter wells and transduced with the viral vector using different moi values as indicated. Cells were harvested 2 days later and protein extract was prepared by freeze–thawing. tk activity was determined by phosphorylation of [^{3}H]acyclovir as described (8). (*B*) GCV susceptibility of ADV/RSV-tk transduced C_6 glioma cells. Duplicate plates of C_6 glioma cells were incubated for 6 hr with ADV/RSV-tk at moi values of 0–1000. After transduction, the cells were treated with either PBS (solid bars) or GCV (hatched bars) at a concentration of 10 μg/ml. Sixty-eight hours later, the surviving cells were counted and percentages of cell survival were calculated by comparing cell counts with those of cultures of nontransduced cells treated with PBS.

Regression of C_6 Glioma in Brains of Nude Mice After *in Vivo* Gene Therapy. In view of the GCV susceptibility of HSV-tk-expressing tumor cells *in vitro*, we tested whether direct transduction of C_6 gliomas *in vivo* followed by GCV treatment would cause tumor regression. Athymic nude mice were injected intracerebrally with 1×10^4 C_6 glioma cells. The gliomas grew rapidly in the caudate nucleus, invaded the cerebral cortex, and spread through the injection needle track into the subgaleal space of the scalp. Untreated animals survived an average of 23 days after tumor cell injection. Eight days after tumor cell injecti n, when the tumors were $\approx$0.4 mm^2 in cross-sectional area, 3×10^8 particles of ADV/RSV-tk or ADV/RSV-β-Gal were injected stereotaxically into the tumors. Carbon particles on the injecti n needle were used to mark the injection site and to verify that the viral injection was in the tumor. Twelve hours after viral injection

the animals were treated intraperitoneally twice daily with either GCV or PBS f r 6 consecutive days. F ur treatment groups f five animals each were established: (*i*) ADV/RSV-β-Gal plus PBS (TK−G−); (*ii*) ADV/RSV-β-Gal plus GCV (TK−G+); (*iii*) ADV/RSV-tk plus PBS (TK+G−); and (*iv*) ADV/RSV-tk plus GCV (TK+G+). The remaining animals were sacrificed 20 days after tumor implantation and the brains were examined microscopically. All of the animals in the TK+G−, TK−G+, and TK−G− groups had large cerebral tumors (Fig. 3*A*) that had compressed and infiltrated adjacent brain parenchyma. The tumors were characterized by hypercellularity, nuclear pleomorphism, and focal vascular proliferation (Fig. 3*B*). Necrosis was not observed in any of the tumors but some had microhemorrhages. In contrast, 2 of the 10 animals in the TK+G+ group were tumor-free (Fig. 3*E*) and the other 8 animals had small residual gliomas surrounding the injection tract (Fig. 3*C*). In animals without residual tumor, a small collection of macrophages and erythrocytes was present at the tumor injection site (Fig. 3*F*). Computerized morphometric analysis of the maximal cross-sectional area of the tumor revealed a 23-fold difference between the mean of the experimental group (TK+G+) and the mean of the three control groups (Fig. 4). There was a slight, but statistically significant ($P < 0.001$), reduction in the mean cross-sectional area of the TK−G+ group when compared to the TK−G− and TK+G− groups, suggesting that GCV treatment alone may have had an inhibitory effect on tumor growth but insufficient to cause tumor regression (Fig. 4).

FIG. 3. Photomicrographs of coronal sections of brains from treated and untreated mice. (*A*) An animal treated with TK+G− showing the presence of a large tumor (outlined by arrowheads). (*B*) Higher magnification of the tumor in *A* showing the carbon particles used to mark the adenovirus injection site. (*C*) An animal treated with TK+G+ showing a small residual tumor along the needle tract (arrow). (*D*) Detail of the residual tumor at the site indicated by the arrow in *C* showing carbon particles. (*E*) An animal treated with TK+G+ with no residual tumor. (*F*) Detail of injection site in *D* showing erythrocytes, macrophages, and carbon particles at the site marked by the arrow in *E*. (*A*, *C*, and *E*, bars = 1 mm; *B*, *D*, and *F*, bars = 50 μm.)

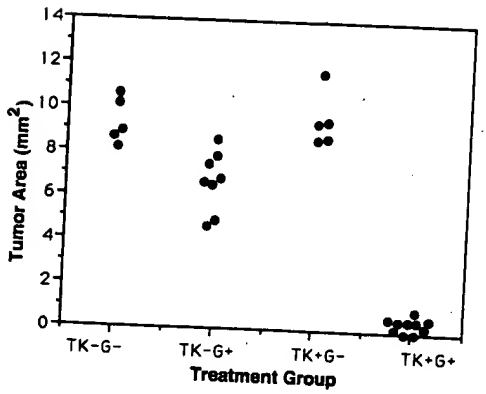

FIG. 4. Cross-sectional areas of tumors in the four experimental groups: (*i*) ADV/β-Gal plus PBS (TK−G−); (*ii*) ADV/β-Gal plus GCV (TK−G+); (*iii*) ADV/RSV-tk plus PBS (TK+G−); (*iv*) ADV/RSV-tk plus GCV (TK+G+). Maximal cross-sectional areas of the tumors were measured by computerized morphometric analysis. Mean cross-sectional area of the TK+G+ group was 0.36 ± 0.33 mm² ($n = 10$) compared to 6.66 ± 1.39 mm² ($n = 8$) for the TK−G+ group, 9.584 ± 1.24 mm² ($n = 5$) for the TK+G− group, and 9.268 ± 1.00 mm² ($n = 5$) for the TK−G− group.

HSV-tk Transduced Normal Brain Cells Are Refractory to GCV Toxicity. To determine whether ADV/RSV-tk and GCV had any deleterious effect on normal brain tissue, we examined coronal sections remote from the tumor site in each animal. In addition, ADV/RSV-tk and GCV were administered to two nude mice without tumors and their brains were examined. No necrosis, demyelination, loss of neurons, ependymal damage, or inflammatory response was observed remote from the tumors and/or needle tracks.

DISCUSSION

These experiments demonstrate that recombinant adenoviral vectors can be used as an efficient gene delivery vehicle for treatment of a localized tumor *in vivo*. The therapeutic significance of these experiments is better appreciated by calculating the volume of the tumors from the maximal measured cross-sectional areas. Assuming that the tumors were spherical, the TK+G+ animals had tumors with a mean volume of 0.054 mm³, whereas the control groups had a mean tumor volume of 28.2 mm³. Based on counting nuclei in representative sections and computing mean nuclear density, the mean number of tumor cells in the TK+G+ group was 5.4 × 10⁴, and the number of tumor cells in the control groups was 2.8 × 10⁷, indicating that the gene therapy resulted in a >500-fold, or 2.7 log, tumor cell reduction.

In previous experiments (9–11) in which 9L gliomas in syngeneic rats were treated with a retroviral vector carrying the HSV-tk gene and then GCV, the tumors were necrotic and were invaded by macrophages and lymphocytes, suggesting a contribution of the cellular immune system in tumor regression. In our experiments, athymic nude mice were used and the tumor cells were destroyed without apparent involvement of the cellular immune response. The transfer of phosphorylated GCV from virally transduced to nontransduced tumor cells via gap junctions has been hypothesized to contribute to tumor regressi n and has been termed the bystander effect (8–12). In our experimental model, this contributi n may be min r since few gap junctions are reported to exist between C_6 cells (17, 18). Thus, the effectiveness of the recombinant aden viral vector in ur experiments appears to be attributable to its high efficiency in

HSV-tk gene transduction of the tumor cells *in vivo*. Indeed, X-Gal staining indicated that ≈50% of the C_6 cells were transduced in the tumors when they were injected with 3×10^8 particles of ADV/RSV-β-Gal and the β-Gal-positive cells were more concentrated along the needle track (data not shown).

It should also be stated that the viral dose used in this study has not yet been optimized. While 3×10^8 particles are sufficient to cause regression of brain tumors of 0.3–0.5 mm, it is not clear yet whether lower viral doses will be equally efficacious. Nevertheless, the present results do illustrate the scientific principle that recombinant adenovirus-mediated delivery of the HSV-tk gene and GCV treatment cause glioma regression *in vivo*. Further studies will certainly include development of better methods for viral vector delivery so that larger tumors can also be treated.

The recombinant adenoviral vector has been extensively used to obtain high levels of transduction and gene expression in cultured mammalian cells and in animals. Adenoviral vectors have a broad host range (19–22) and have been used to produce live viral vaccines (21) and for gene therapy in cystic fibrosis (23). In principle, only dividing cells are damaged by phosphorylated nucleoside analogues. Neurons are postmitotic and should not be damaged by this form of therapy. However, astrocytes, oligodendrocytes, ependymal cells, and vascular endothelium do undergo cell division and could be vulnerable to phosphorylated GCV. Injection of the brain with adenovirus vector bearing a β-Gal gene results in efficient transduction of the ependyma and choroid plexus and transduction of neurons, astrocytes, and oligodendrocytes to a lesser extent (24–27). When ADV/RSV-tk was injected into brains of nude mice and GCV was administered, no apparent damage to the tissue was observed other than that resulting from the needle insertion (data not shown). Initial results in a separate experiment demonstrated that some TK+G+ animals survived >50 days after tumor cell inoculation and died because of secondary tumors (data not shown), while control animals all died from the primary brain tumors by 23 days. Thus, the procedure appears to have the potential for long-term efficacy. Nevertheless, long-term studies of possible glial, endothelial, and neuronal toxicity must be performed as well as survival studies to assess the potential of recombinant adenoviral-mediated gene therapy of brain tumors.

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